AMES ASSAY TEST RESULTS OF WELL WATER IN THE WHITCHURCH-STOUFFVILLE AREA

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SUMMARY

A study was initiated on five ground water wells in the Whitchurch-Stouffville area to determine 1) if water concentrates from these wells could induce changes in the genetic structure of cells (i.e. were mutagenic) when tested by the Ames Salmonella assay and 2) if seasonal variation has influenced the mutagenic classification of these wells. The five wells included two private wells adjacent to York Sanitation Landfill site No. 4, an observation well on the landfill and two private wells which could not be affected by the landfill based on hydrogeologic considerations.

State of the art technology in sampling, concentrating and extracting the water was utilized in the preparation of each concentrate. A 20 litre concentrate was prepared from each well and a 400 L (approximate volume) concentrate was prepared from all but the on-site observation well. Because the observation well lacked a system to pump water out, it was difficult to withdraw samples much larger than 20 L.

Each concentrate was tested for both toxicity and mutagenicity. Toxicity of less than one log kill was noted with one of the concentrates. This level of toxicity is generally considered to be insignificant relative to the mutagenic classification of the sample tested. The mutagenic property of each concentrate was evaluated with the Ames Salmonella/mammalian microsome plate incorporation assay. All concentrates produced non-mutagenic results confirming the negative results reported in the November 1982 Whitchurch-Stouffville mutagenicity study.

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INTRODUCTION

In November 1982, a MOE report was released in which Ames mutagenicity data for six wells in the Whitchurch-Stouffville area were presented. It was generally concluded that all well waters were non-mutagenic. However, the report recommended that three of the wells (Ballantrae Plaza, Hutchinson and Fockler) be retested as some of the data for these wells, in one lab, were ambiguous. In addition, it was recommended that a quantity of water significantly greater than 20 L be taken from each well and that these samples be taken in a season other than winter. In the fall of 1982, this Ministry initiated a study of well water in the Whitchurch-Stouffville area to address these recommendations. The results of the study are presented here.

OBJECTIVES

The prime objectives of the study were the following:

1. To test a 20 L sample from the Ballantrae Plaza, Hutchinson and Fockler wells, as well as a control well, for mutagenicity using the Ames assay, and to compare the results with those of an earlier study.
2. To test, using the Ames assay, a second sample of approximately 400 L from each of the above four wells.
3. To test for mutagenicity a 20 L sample from an observation well located on the landfill site.
4. To compare all mutagenicity data obtained here with results of the initial tests carried out in October 1981 and those reported in November 1982 with special reference to possible seasonal variations in the results.

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METHODS

Wells Tested

Five wells in the Whitchurch-Stouffville area were selected for this test program. All five wells are in the general area of the York Sanitation #4 landfill site. Three of the wells, Ballantrae Plaza, Fockler and Hutchinson were chosen to comply with recommendations contained in the November 1982 Whitchurch-Stouffville mutagenicity study final report. A fourth site, Consolidated Gravel, was chosen as it is both up-gradient in the main aquifer system which underlies the landfill site and adjacent areas, and available chemical monitoring data suggest it contains uncontaminated water. The fifth site, observation well OW 16-70, is located within the boundaries of the landfill area and is used for monitoring the main underlying aquifer.

Samples Tested for Mutagenicity

1. Reagent Blank

The reagent blank provided a control on the extraction and concentration procedures, especially the possible induction of revertants by the reagents. A randomly selected XAD-2 cartridge (see November 1982 report) was washed with 1 litre Super Q (see definition below) water and then dried for 10 minutes in a stream of N_2 gas, the cartridge was then extracted with three 50 mL volumes of acetone, and further extracted with three 50 mL volumes of methylene chloride. All elution solvents were of "distilled in glass" purity.

The extract was concentrated by rotary evaporation to a volume of 20-30 mL. This volume was then sub-divided into two portions. Three-fourths was set aside for biological testing and this portion was further reduced by rotary evaporation until all elution solvent was removed. The resultant 4-5 mL aqueous

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volume was then brought back up to 15 mL with DMSO and stored at -60° C until tested. The one-fourth portion was stored in the methylene chloride at -60°C until needed.

2. Super Q Blank

The Super Q blank served as a pure water control assuring the absence of elutable background mutagenic activity from the XAD-2 resin cartridges. Two XAD-2 cartridges were selected at random and were processed in the field along with the cartridges used to collect the sample. After the initial 1 litre wash, 20 litres of Super Q water (i.e., distilled water passed through a millipore (Millipore Corp.-Belford, Mass.) deionization-filtration system, consisting of one carbon filter (CDFC-02203-3), 2 ion columns (CDMB-02202) in parallel, and a 0.45 µ millitube micro filter) was passed through each cartridge. These cartridges were then returned to the lab, their eluates pooled and further processed as was the reagent blank.

3. 20 Litre Sample

A 20 litre water concentrate was obtained from each well. This concentrate was comparable to the highest concentrate tested in the 1982 mutagenicity study of these wells and other wells in this area. Water from the four private wells was collected via household faucets, while observation well OW 16-70 had to be baled as there was no pumping system. The water sample was passed through a single XAD-2 cartridge at a flow rate which varied from well to well but did not exceed 80 mL/minute.

As with the reagent and Super Q blanks, the 20 litre cartridge was dried under a N_2 stream, and eluted with three 50 mL volumes each of acetone and methylene chloride. The eluate was rotary evaporated down to approximately 25

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mL and this volume was split 3:1 into two fractions. Both fractions were vortex concentrated down to 3-5 mL and about 1 mL aqueous volume respectively, and each then brought up with DMSO to working volumes of 15 and 5 mL, respectively. The 15 mL concentrate was subsequently tested for mutagenicity.

4. 400 Litre Sample

The major sample from each source was approximately 400 L affording an approximate 10 fold increase in concentration over previous tested samples. The sample was taken by using 8 cartridges attached in parallel. A single manifold serviced all cartridges and provided a relatively uniform distribution of water sample to each cartridge. The flow ratio of each cartridge varied slightly within and between individual well cartridge groups but in all cases flow rates did not exceed 75 mL/minute/cartridge.

Each cartridge was returned to the laboratory and extracted as described for the reagent, Super Q and 20 litre cartridges. The eluates were combined and concentrated to 10 mL by Kuderna Danish evaporation. The concentrate was divided 3:1 into two portions ($7\frac{1}{2}$ and $2\frac{1}{2}$ mL, approximately). The $2\frac{1}{2}$ portion was vortex evaporated to an approximate 1 mL aqueous solution and then brought up to 10 mL with DMSO. This concentrate was subsequently tested for mutagenicity. The $7\frac{1}{2}$ mL portion was vortex concentrated to approximately 3 mL, brought back to 30 mL with DMSO and then stored at -60°C.

5. Positive Control Substances

Like the Reagent and Super Q blanks, the positive control substances serve as controls on the mutagenicity test system. Positive control substances monitor the tester strains ability to be mutated. In addition, they also provide a check on the viability of the S9 activation system.

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Expression of Doses as Equivalent Volumes of Unconcentrated Water Sample

The doses tested in the Salmonella assay are equivalent to a given volume of the water sampled from each well. The table below lists the doses tested and indicates their equivalent volume relative to the original volume of water sampled.

Dose tested (mL) Concentrate	Equivalent volume (mL) relative to each well water sample volume (L)			
Sample Volume -	20 L	337 L	400L'	447 L
0.02	20 mL	169 mL	200 mL	224 mL
0.05	50 mL	422 mL	500 mL	559 mL
0.10	100 mL	843 mL	1000 mL	1118 mL
0.20	200 mL	1685 mL	2000 mL	2235 mL

' The 400 L equivalent values closely approximate those achieved for the 404 and 408 L samples.

Toxicity Check

A nutrient broth culture of Salmonella typhimurium was diluted in nutrient broth to titres of 10^3 (Dilution 1) and 10^4 (Dilution 2) cells per mL. A 0.1 mL volume of diluted cell suspension was mixed in 13 x 100 mm test tubes with 2.0 mL molten top agar at 42°C. Tubes of inoculated top agar were overlaid on replicate nutrient agar plates. These plates were incubated overnight at 37°C. Colonies developing on these plates were counted and these counts recorded as control bacterial numbers.

To determine bacterial toxicity, a volume of sample was mixed with top agar and inoculated with the diluted cell suspension. The volume of sample

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tested was equivalent to the largest volume of sample tested in the Salmonella assay. Top agar containing the cell suspension and the sample were mixed and then overlaid on a nutrient agar plate. Colonies developing on these plates were determined in a manner identical to that used for the control plates.

To evaluate the toxicity, bacterial counts on control plates were averaged and compared to the number of colonies counted on plates containing sample. A decline in the numbers of colonies on toxicity plates of more than 1 log is generally considered to be necessary before a significant impact on the mutagenicity results will occur.

It should be noted that toxicity observed towards Salmonella cells does not necessarily equate to toxicity in other biological systems.

Mutagenic Tests

All samples were tested on the Ames' Salmonella/mammalian microsomal mutagenicity test using the plate incorporation method. The procedures associated with this method are presented in the MOE laboratory's Standard Operating Procedure (SOP), which is attached as an Appendix.

The tester strains used for this study were TA 1535, TA 1537, TA 97, TA 98 and TA 100. In the early tests the newly engineered tester strain, TA 97, was used in place of TA 1537. TA 97 is, in essence, TA 1537 with the addition of the pkm 101 episome. Results from these initial tests indicated that the spontaneous revertant rate for TA 97 was too high and lacked uniformity. Therefore, TA 97 was replaced by TA 1537 and all initial tests rerun on TA 1537.

All test and control plates were scored using a standard dissection microscope set at a 70X magnification.

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All plates at the highest doses were routinely checked under 200X magnification to determine the presence of a normal background bacterial lawn. This check provided both an assurance that the colonies were truly mutants, rather than artifacts of toxicity, and a measure of the toxic effect, if any.

Presentation of Data

The data are presented in Tables as the average number of revertant colonies on 3 replicate plates rounded off to the nearest whole number.

In each Table the tester strains are designated by their last two numbers, i.e. TA 1535 is designated as 35, TA 98 is designated as 98, and so on.

Criteria for a Positive Mutagenic Response

The evaluation of a positive mutagenic response was based upon three criteria. First, the average revertant colony number on a given set of test plates must exceed that of spontaneous revertant controls plates by at least 2.5 times. This also assumes that the spontaneous revertant number is within the normal range for that strain tested and that the revertant colony number observed for the Reagent or Super Q blanks is not exceedingly high. Second, there should be a positive correlation between the dose administered and the observed response, such that there is a demonstrable dose-effect response. Finally, the observed response must be repeatable.

Three positive control substances were used in this study: 2-nitrofluorene (2NF), sodium azide (NaN_3) and 2-aminoanthracene (2AA). 2NF is a direct acting mutagen which should produce a positive mutagenic response on strains TA 1537 and TA 98 in the absence of S9. In general, 2 g will mutate TA 98 while 20 g is required for an adequate response from TA 1537. NaN_3 is another

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direct acting chemical which will mutate TA 1535 and TA 100. In this study a 1 µg dose of NaN_3 per plate was used. Neither 2NF or NaN_3 require S9 to function as mutagens.

The third substance, 2AA, is a promutagen and therefore requires some metabolic configurational alteration in order to induce mutations. This metabolism is provided by the S9 activation or metabolic enzyme system. Once activated, 2AA, at a dose of 1 µg/plate, should mutate all four tester strains. Without activation there should be no mutagenic response with this substance.

The positive response of a tester strain to either a direct acting or promutagen is sufficient to consider the tester strain mutagenically sensitive or active. Therefore, in those cases where the strain responded differently to two different mutagens, it can be assumed that there has been an improper dosing of the mutagen that exhibited the negative response. Where possible, however, to ensure the validity of the results, the tester strain in question was rechecked both with the positive control substance as well as the test concentrate.

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RESULTS

Well Location and Sampling Information

The location of the five wells sampled during this study is presented in Figure 1. Of the five wells, the Ballantrae Plaza and Consolidated Gravel are the most removed from the immediate area of the landfill. The Fockler and Hutchinson wells are located on private property adjacent to the landfill. The OW 16-70 well is located within the boundaries of the landfill and earlier chemical surveys had indicated that it contains more organic material than the four off-site private wells included in this study.

The date of sampling, as well as the types of samples taken from each well is provided below. All wells were sampled during a two-week period near the end of October, 1982.

Well Site	Sample Date	Samples Taken
Ballantrae Plaza	October 22/82	20 L, 337 L, Super Q, RB[1]
Consolidated Gravel	October 26/82	20 L, 404 L, Super Q
Fockler	October 19/82	20 L, 408 L, Super Q
Hutchinson	October 28/82	20 L, 447 L, Super Q, RB[1]
OW 16-70	November 4/82	20 L, Super Q, RB[1]

[1]RB - Reagent Blank.

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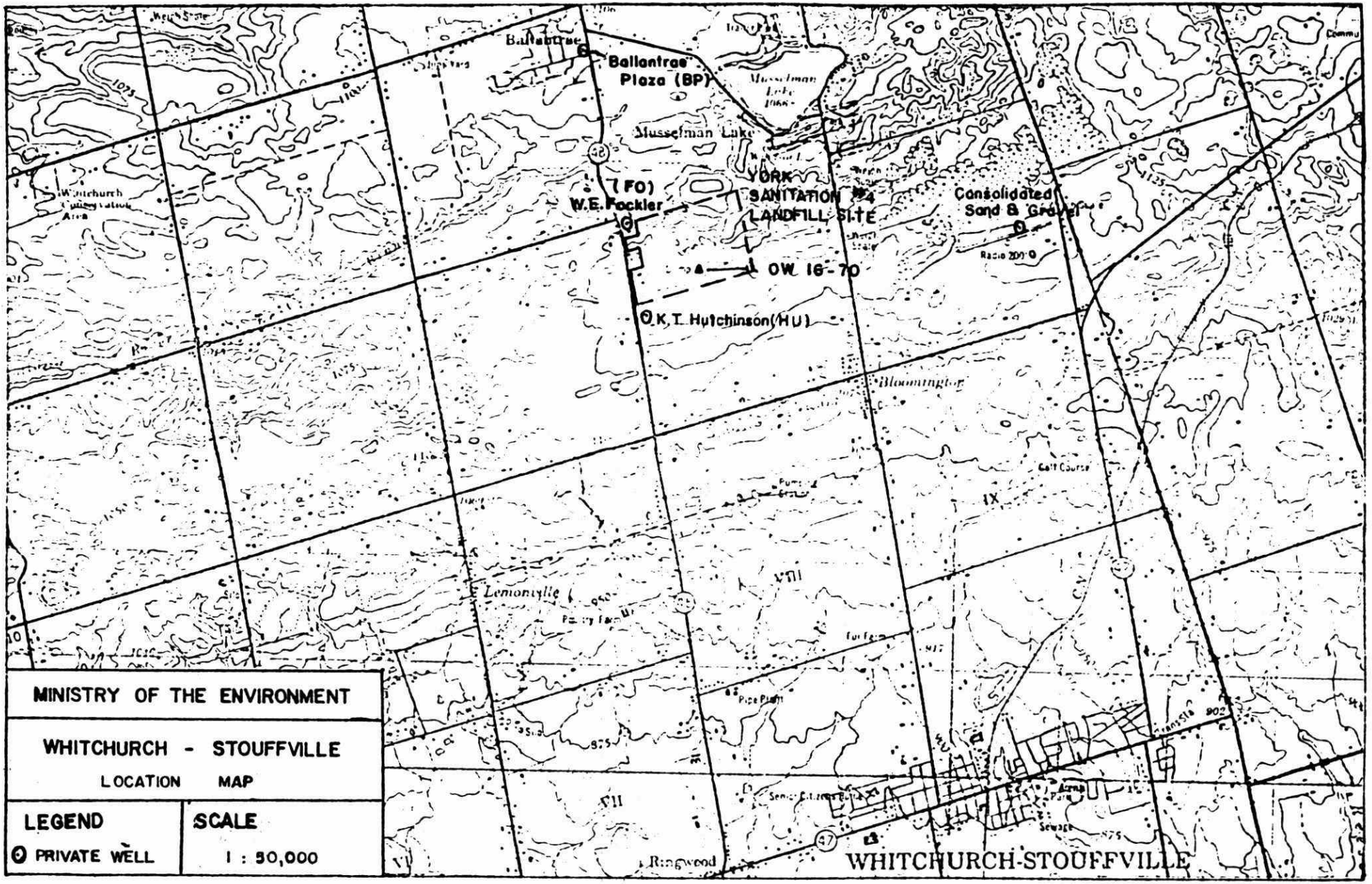

Figure 1. Location of wells sampled.

Quality Assurance Tests

A. Spontaneous Mutation Rate

The spontaneous mutation rates for tester strains TA 1535, TA 1537, TA 98 and TA 100 were each consistently in the normal range throughout the testing of all the wells. The spontaneous rate for TA 97, however, was significantly greater than expected, therefore, TA 97 was dropped in favor of parent strain TA 1537. The concentrates initially tested with TA 97 were retested with TA 1537. Specific spontaneous mutation rates observed during the testing of each well are provided below with the results from the individual wells.

B. Super Q Concentrate

Results of the mutagenicity tests on the pure water concentrates indicated that these concentrates did not induce mutations in Salmonella. The test data from the individual wells, provided below, showed some variation but never significantly exceeded the spontaneous mutation rate.

C. Reagent Concentrate

Mutagenicity test results on concentrates from reagent blanks indicated that these concentrates did not significantly increase the mutation frequency in Salmonella. This suggests that the solvents used in the concentration and extraction methodologies did not affect the mutagenicity data. A detailed account for the individual reagent blanks is provided below with the results of the Ballantrae Plaza, Hutchinson and OW 16-70 tests. Reagent blanks were not run with samples from the Fockler and Consolidated Gravel wells.

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D. Positive Control Substances

The three positive control substances (2NF, NaN_3 and 2AA), with few exceptions, produced consistently positive data. In the few cases, where the data were non-positive for a particular substance, the problem could be isolated to the particular solution prepared or to a weakly active S9 buffer. In every case, however, where there was any inconsistency in the positive control data, the test was repeated to assure the validity of the results.

Individual Well Data

A. Consolidated Gravel

1. Toxicity

A 0.2 mL sample of the 20 litre concentrate was non toxic to either dilution 1 or dilution 2 of Salmonella tester strain TA 98; however, 0.2 mL of the 404 litre sample did produce a slight toxic effect (Tables 1 and 4). The toxicity produced was less than a 1 log kill and therefore should not have affected the mutagenicity test results.

2. Spontaneous Mutation Rate

During the testing of all the Consolidated Gravel associated samples, a total of three spontaneous mutation rate checks were run on all the tester strains (Table 2, 3 and 5). The consistency between these checks was fairly good for all the strains, and the range of the spontaneous mutation frequency was well within the normal range for each particular strain.

3. Super Q Concentrate

The Super Q concentrate was tested twice, due to the lower than anticipated response in the first test with the positive control substance 2AA with

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TABLE 1

Toxic Effect of the Consolidated Gravel 20 Litre Water Concentrate on Two Dilutions of Salmonella TA 98 Cells.

Volume of Concentrate Tested	Mean and Standard Error of Number of Surviving Colonies	
	Dilution 1	Dilution 2
0.00 mL	27.8 ± 0.5	302 ± 16
0.05	30.0 ± 0.6	317 ± 19
0.20	17.0 ± 1.5	386 ± 23

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TABLE 2

Mutagenicity Test Data for Super Q Blank and 20 L Water Concentrate from Consolidated Gravel. Each Value Represents the Average Revertant Colony Number from 2 - 3 Replica Plates.

Spontaneous Reversion Numbers (0) and Positive Control Data are Included.

Sample	Tester Strains (+ or - S9)							
	35		37		98		100	
	-	+	-	+	-	+	-	+
0	25	16	3	2	19	16	193	190
Positive Standards								
2NF (2 µg)					393			
2NF (20 µg)			11					
NaN_3 (1 µg)	477							
2AA (1 µg)	24	26	2	2	11	20	182	179
Super Q Blank								
0.05 mL	24	16	2	6	16	16	153	178
0.20	22	14	1	3	18	16	178	187
20 L Concentrate								
0.05 mL	21	17	3	5	17	22	192	183
0.10	28	14	1	3	17	16	194	173
0.20	26	28	3	3	21	19	165	171

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TABLE 3

Repeat of Mutagenicity Test Data for Super Q Blank and 20 L Concentrate from Consolidated Gravel. Each Value Represents the Average Revertant Colony Number from 2 - 3 Replica Plates.

Spontaneous Reversion Number (0) and Positive Control Data are Included.

Sample	Tester Strains (+ or - S9)							
	35		37		98		100	
	-	+	-	+	-	+	-	+
0	23	14	2	3	18	29	127	130
Positive Standards								
2NF (2 µg)					582			
(20 µg)			16					
NaN_3 (1 µg)	591						533	
2AA (1 µg)	32	69	1	13	16	422	158	868
Super Q Blank								
0.05 mL		22		2		12		130
0.20		23		1		9		158
20 L Concentrate								
0.05 mL		29		2		12		145
0.10		26		2		11		122
0.20		18		2		10		109

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TABLE 4

Toxic Effect of the Consolidated Gravel 404 Litre Water Concentrate on a Single Dilution of Salmonella TA 98 Cells.

Volume of Concentrate Tested	Mean and Standard Error of Number of Surviving Colonies
0.00 mL	55.8 ± 6.6
0.05	56.7 ± 2.6
0.20	17.3 ± 1.9

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TABLE 5

Mutagenicity Test Results for the Consolidated Gravel 404 L Water Concentrate.
Each Value Represents the Average Revertant Colony Number from 3 Replicate Plates.

Spontaneous Reversion Number (0) and Positive Control Data are Included.

Sample	Tester Strains (+ or - S9)							
	35		37		98		100	
	-	+	-	+	-	+	-	+
0	15	11	3	3	10	21	134	156
Positive Standards								
2NF (2 µg)					276			
2NF (20 µg)			22					
NaN_3 (1 µg)	771						887	
2AA (1 µg)	15	159	1	47	13	956	146	1411
20 L Concentrate								
0.02 mL	14	12	-	-	8	24	133	183
0.05	13	11	2	4	17	23	130	153
0.10	12	10	1	3	13	17	132	160
0.20	13	11	2	2	15	19	155	153

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all four strains in the presence of S9. In the initial test, the 0.05 mL sample exhibited an unexpected elevated response with tester strain TA 1537 in presence of S9; however, the 0.2 mL sample did not show an elevated revertant response under the same circumstances (Table 2). The values observed for TA 1537 in the absence of S9 and for all other tester strains were within the limits for the spontaneous mutation rate obtained that day for each strain. When the concentrate was retested in the presence of S9 all of the revertant counts for any strain including TA 1537 were within the spontaneous rate range observed for that day (Table 3).

4. Reagent Concentrate

No reagent concentrate was prepared with this sample.

5. Positive Control Substance

Three checks were made on the response of the tester strains to mutagenic substances. In each case 2NF produced positive mutagenic responses with strains TA 1537 and TA 98 in the absence of S9 (Tables 2, 3 and 5). Similarly, NaN_3 induced positive mutagenic responses with strains TA 1535 and TA 100 in the absence of S9 (Tables 2, 3 and 5). These results indicate that all four tester strains were sensitive to mutagenic substances and therefore should give a positive response in the presence of an appropriate mutagen. The check of the ability of the S9 to convert the promutagen 2AA into a mutagen produced negative results for all four strains (Table 2). Therefore, all the S9 data were suspect. When repeated (Tables 3 and 5), 2AA in the presence of S9 produced the expected positive mutagenic result with all four strains.

6. 20 L Sample

As with the Super Q concentrate, the 20 litre concentrate was tested twice. In both trials the three doses tested produced non-mutagenic responses with

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all four tester strains, although in the initial test there was a slightly elevated response for the 0.05 mL dose with TA 1537 plus S9 (Tables 2 and 3).

7. 404 L Concentrate

Four doses of the concentrate from the 404 litre sample were tested with each tester strain. In each case the number of induced revertant colonies observed did not significantly exceed that of the spontaneous mutation rate obtained for the given tester strain (Table 5). These data support the non-mutagenic response observed for the 20 litre sample.

B. Ballantrae Plaza

1. Toxicity

When tested against two dilutions of TA 98, neither the 0.05 mL nor the 0.20 mL dose of the 337 litre concentrate produced any toxic effect (Table 6). Since the maximum concentration to be tested mutagenically did not exhibit toxic properties, a toxicity check on the less concentrated 20 litre concentrate was not performed.

2. Spontaneous Mutation Rate

In each of the three checks on the spontaneous mutation rate, the rate observed was within the normal range for that respective strain (Table 7, 8 and 9). Strain TA 1535 elicited the greatest amount of variation between the three checks, but in all cases the revertant colony numbers obtained for the Super Q, reagent, 20 L and 337 L concentrates tested against TA 1535 reflect the same variation (Tables 7, 8 and 9).

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TABLE 6

Toxic Effect of the 337 Litre Water Concentrate Obtained from the Ballantrae Plaza. The Toxicity is Measured Against 2 Dilutions of Salmonella TA 98 Cells.

Volume of Concentrate Tested	Mean and Standard Error of Number of Surviving Colonies	
	Dilution 1	Dilution 2
0.00 mL	19.4 ± 2.2	214 ± 19
0.05	24.3 ± 0.3	273 ± 9
0.20	25.0 ± 8.4	268 ± 4

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3. Super Q Concentrate

Both doses of the pure water concentrate tested against all four tester strains produced non-mutagenic results (Table 7). The revertant numbers with TA 1537 were higher than observed for the spontaneous rate but were not significantly higher. The failure of 2AA to produce a significantly elevated revertant for TA 1537 in the presence of S9 (Table 7a) necessitated a repeat test of the 2AA, Super Q and Reagent concentrate with TA 1537. The results of the retest indicate a definite non-mutagenic response (Table 7b).

4. Reagent Concentrate

The response of the reagent blank concentrate paralleled that of the Super Q even to the values obtained with TA 1537 (Table 7a). The repeat test clearly shows a non-mutagenic response (Table 7b).

5. Positive BP Control Checks

For the most part the mutagenic control substances were detected as positive by all the strains in each of the three checks performed with BP water samples. There were, however, two exceptions. 2AA produced only a very weak response with TA 1537 during the testing of the blanks (Table 7) and TA 98 did not respond to 2NF (Table 9). Since TA 1537 responded well to 2NF and 2AA was able to elicit positive responses from all other strains, the reason for the lack of a stronger TA 1537 - 2AA mutagenic activity is unclear. When 2AA was retested with TA 1537 the result was strongly mutagenic (Table 7b). The 2NF data in Table 9a necessitated a repeat test with TA 98. These results indicated a strong mutagenic response for 2NF while confirming the non-mutagenic response of the 337 L concentrate on TA 98 (Table 9b).

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TABLE 7a

Ballantrae Plaza Spontaneous Revertant Rate Positive Control Data and Test Results on the Super Q and Reagent Blanks.
Each Value Represents the Average Revertant Colony Number from 3 Replicate Plates.

Spontaneous Reversion Number (0) and Positive Control Data are Included.

Sample	Tester Strains (+ or - S9)							
	35		37		98		100	
	-	+	-	+	-	+	-	+
0	33	13	4	3	21	27	177	178
Positive Standards								
2NF (2 µg)					990			
2NF (20 µg)			11					
NaN_3 (1 µg)	643						767	
2AA (1 µg)	30	73	2	7	16	319	176	785
Super Q Blank								
0.05 mL	21	18	4	5	18	19	176	179
0.20	20	14	3	6	18	19	189	172
Reagent Blank								
0.05 mL	23	18	2	5	19	19	152	179
0.20	22	13	2	6	15	19	178	172

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TABLE 7b

Retest of the Ballantrae Plaza Super Q and Reagent Concentrates with TA 1537.

Each Value Represents the Average Revertant Colony Number from Three Replica Plates.

Sample	Tester Strain (+ or - S9)	
	TA 1537	
	-	+
0	4	8
Positive Standards		
2NF - 20 µg	138	-
2AA - 1 µg	5	123
Super Q		
0.05	4	2
0.20	5	5
Reagent Blank		
0.05	2	5
0.20	4	6

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TABLE 8

Mutagenicity Test Results for the Ballantrae Plaza 20 L Water Concentrate.
Each Value Represents the Average Revertant Colony Number from 3 Replicate Plates.

The extra TA 100 minus S9 column is a repeat experiment - see text.

Spontaneous Reversion Number (0) and Positive Control Data are Included.

Sample	Tester Strains (+ or - S9)								
	35		37		98		100		100
	-	+	-	+	-	+	-	+	-
0	34	18	3	3	26	33	203	173	201
Positive Standards									
2NF (2 µg)					582				
2NF (20 µg)			16						
NaN_3 (1 µg)	536						-		1450
2AA (1 µg)	31	100	3	34	18	728	166	827	-
20 L Concentrate									
0.05 mL	35	16	2	1	32	32	182	179	226
0.10	42	20	5	3	25	29	180	173	241
0.20	35	29	0	0	24	25	192	172	231

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TABLE 9a

Mutagenicity Test Results for the Ballantrae Plaza 337 L Water Concentrate.
Each Value Represents the Average Revertant Colony Number from 3 Replicate Plates.

Spontaneous Reversion Number (0) and Positive Control Data are Included.

Sample	Tester Strains (+ or - S9)							
	35		37[1]		98		100	
	-	+	-	+	-	+	-	+
0	17	8	3	3	13	19	171	166
Positive Standards								
2NF (2 µg)					19			
(20 µg)			22					
NaN_3 (1 µg)	537						783	
2AA (1 µg)	16	115	1	13	17	954	146	881
20 L Concentrate								
0.02 mL	20	11	-	-	15	22	188	180
0.05	14	15	2	4	21	22	161	175
0.10	26	8	3	5	21	23	158	129
0.20	21	10	2	3	10	22	146	135

[1] The tests with TA 1537 were performed as a replacement for the non-acting TA 97 and therefore were not run concomitant with those for the other three strains.

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TABLE 9b

Repeat Test of Ballantrae Plaza 337 L Sample on TA 98

Sample	Tester Strain
	98 minus S9
0	23
Positive Control	
2NF - 2 µg	982
337 L Concentrate	
0.02 mL	22
0.05	21
0.10	25
0.20	24

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6. 20 L Concentrate

None of the doses for the 20 L concentrate tested produced a mutagenic response with any of the tester strains (Table 8). The NaN_3 check on TA 100 minus S9 was inadvertently omitted. Even though the 2AA response with TA 100 indicated that the strain was sensitive, the strain was retested. These data, which include the NaN_3 check, are presented in Table 8 as a separate column and again indicate the concentrate was non-mutagenic.

7. 337 L Concentrate

The four doses of the 337 L concentrate tested all produced non-mutagenic responses with each of the four tester strains (Table 9). None of the induced responses significantly exceeded the spontaneous reversion rates for any of the tester strains. Since, the positive control substance 2NF failed to produce a mutagenic response with TA 98, the TA 98 data were repeated (Table 9b). In the repeat test, 2NF produced a very strong mutagenic response while the 337 L concentrate was clearly negative.

Hutchinson Well

1. Toxicity

No toxicity check on the 20 L or 447 L sample were performed due to insufficient sample at the time of the experiment. Toxicity was however monitored by scrutinizing the lawn of most 447 L mutagenicity test plates. No evidence of toxicity was observed during these checks.

2. Spontaneous Revertant Rate

The spontaneous reversion rate elicited normal fluctuations within the expected range for each strain (Tables 10a, 11 and 12a). TA 1535 in Table 10a

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and TA 98 in Table 12a both produced spontaneous rates which were low relative to the other two checks for those strains, whereas that for TA 100 (Table 11) was relatively high.

3. Super Q Concentrate

Relative to the spontaneous mutation rates for the tester strains, the pure water induced revertant rate for tester strains TA 1537, TA 98 and TA 100 was not significant (Table 10a). The testing of the Super Q blank on TA 1535 was performed on a different day from that of the other three strains. The results indicate that the concentrate was also non-mutagenic on TA 1535 (Table 10b).

4. Reagent Concentrate

The data for the reagent blank concentrate indicates a non-mutagenic response for both doses with all four strains (Table 10a). The 0.20 mL dose in the presence of S9 did not produce any revertant colonies on strain TA 1535. Upon examination of the plates it was noted that the lawn was missing suggesting a lack of cells. Therefore that portion of the test was repeated. The repeat results indicate a non- positive response (Table 10b).

5. Positive Control Substance

The positive control substances responded as expected. NaN_3 produced mutagenic responses with TA 1535 and TA 100 in the absence of S9, while 2NF did the same for TA 1537 and TA 98 (Tables 10a, 10b, 11 and 12). The 2AA check on the activity of the S9 enzymes indicated that the S9 enzymes were functional (Tables 10a, 10b, 11 and 12).

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TABLE 10a

Mutagenicity Test Results Obtained for the Super Q and Reagent Blanks Prepared Concomitantly with the Hutchinson Water Concentrates.
Each Value Represents the Average Revertant Colony Number from 2 or 3 Replica Plates.

Spontaneous Reversion Number (0) and Positive Control Data are Included.

Sample	Tester Strains (+ or - S9)							
	35		37[1]		98		100	
	-	+	-	+	-	+	-	+
0	13	9	2	4	27	28	165	221
Positive Standards								
2 NF (20 µg)			14		900			
NaN_3 (1 µg)	459						612	
2AA (2 µg)	17	71	3	25	22	507	146	871
Reagent Blank								
0.05 mL	21	16	4	3	21	49	154	174
0.20	19	0	3	3	26	50	174	168
Super Q Blank								
0.05	-	-	2	3	21	52	158	199
0.20	-	-	2	3	19	38	143	172

[1] The test of the Super Q Concentrate on TA 1537 was run at a different time from all other tests reported in this table.

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TABLE 10b

Mutagenicity Test Results for the Hutchinson Well Super Q Concentrate and a Retest of the Hutchinson Reagent Concentrate on Tester Strain TA 1535.[1]

Each Value Represents the Average Revertant Colony Number from Three Replica Plates.

Spontaneous Reversion Number (0) and Positive Control Data are Included.

Sample	Tester Strain (+ or - S9)	
	35	
	-	+
0	39	24
Positive Standards		
NaN_3 (1 µg)	508	
2AA (1 µg)		412
Super Q		
0.02 mL	53	17
0.20	42	14
0	-	19
Positive Standards		
2AA	-	229
Reagent Blank		
0.05	-	18
0.20	-	16

[1] The tests for The Super Q and Reagent concentrate were performed on two different TA 1535 clones, therefore, each has its own spontaneous and positive control values.

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TABLE 11

Mutagenicity Test Results on the Hutchinson 20 L Water Concentrate.
Each Value Represent the Average Revertant Colony Number from 3 Replica Plates.

Spontaneous Reversion Number (0) and Positive Control Data are Included.

Sample	Tester Strains (+ or - S9)							
	35		37		98		100	
	-	+	-	+	-	+	-	+
0	30	20	3	7	26	37	225	224
Positive Standards								
2NF (2 µg)					1109			
2NF (20 µg)			49					
NaN_3 (1 µg)	387						743	
2AA (1 µg)	-	166	-	70	-	901	-	2341
20 L Concentrate								
0.05 mL	28	26	1	6	16	29	193	220
0.10	35	21	5	6	17	31	200	216
0.20	34	23	4	5	13	26	203	186

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TABLE 12a

Mutagenicity Test Results for the Hutchinson 447 L Water Concentrate.
Each Value Represent the Average Revertant Colony Number from 3 Replica Plates.

Spontaneous Reversion Number (0) and Positive Control Data are Included.

Sample	Tester Strains (+ or - S9)							
	35		37		98		100	
	-	+	-	+	-	+	-	+
0	39	24	3	3	19	16	164	172
Positive Standards								
2NF (2 µg)					440			
2NF (20 µg)			22					
NaN (1 µg)	818						837	
2AA (1 µg)	45	130	1	47	12	687	165	1269
20 L Concentrate								
0.02 mL	43	18	-	-	-	-	-	-
0.05	52	20	6	6	21	43	154	149
0.10	37	19	2	5	21	37	178	181
0.20	31	25	2	2	17	32	176	169

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TABLE 12b

Retest of Hutchinson 447 L Concentrate on Strain TA 98

Sample	Tester Strain
	TA 98 + S9
0	25
Positive Control	
2 AA - 1 µg	1957
447 L Concentrate	
0.02	30
0.05	27
0.10	24
0.20	26

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6. 20 L Concentrate

The induced revertant colony results for the 20 L water concentrate clearly indicate a non-mutagenic response (Table 11). None of the observed induced revertant values significantly exceeded their respective spontaneous mutation rate number.

7. 447 L Concentrate

The doses of 447 L concentrate which were tested against strains TA 1535, TA 1537 and TA 100 all yielded definite non-mutagenic responses (Table 12a). With TA 98 in the presence of S9, there is a slight increase over the spontaneous rate in the number of revertants per plate for each of the three doses tested, although there is no clear dose-related response. As mentioned above, however, the spontaneous rate, although within the normal range, is low relative to that observed when the other samples from this well were tested.

However, although these results do not represent a significant positive effect, a second check of the sample with TA 98 plus S9 was made. The results of the retest were clearly negative (Table 12b). All the data for the sample were within the limits of the observed spontaneous mutation rate, while the results for the positve control substance, 2AA, were strongly mutagenic.

Fockler

1. Toxicity

When 0.05 and 0.20 mL of the 408 L concentrate were tested for toxicity, a very slight toxic effect was observed (Table 13). Visual checks indicated that This toxicity did not affect lawn development on the 408 L concentrate mutagenicity plates. No toxicity was performed with the 20 L concentrate.

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TABLE 13

Toxicity Check on the Fockler 408 L. Water Concentrate.
Toxicity Determined on 2 Dilutions of Salmonella TA 98 Cells.

Volume of Concentrate Tested	Mean and Standard Error of Number of Surviving Colonies	
	Dilution 1	Dilution 2
0.00 mL	17.8 ± 2.3	195 ± 18
0.05	18.7 ± 3.2	221 ± 14
0.20	17.0 ± 4.6	167 ± 9

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2. Spontaneous Mutation Rate

The spontaneous mutation rates for all the strains were within the expected range (Tables 14 and 15). TA 1535 had a spontaneous rate slightly lower than seen with previous wells in this study, but still within the acceptable limits for that strain.

3. Super Q Concentrate

The two doses of the pure water concentrate tested clearly produced non-positive results with strains TA 1537, TA 98 and TA 100 (Table 14). With TA 1535 the concentrate induced revertant numbers 1½ to 2 times the spontaneous level. This undoubtedly reflects the observed lower spontaneous rate for TA 1535 rather than an indication of a possible mutagenic response.

4. Reagent Concentrate

No reagent concentrate was prepared with this well sample.

5. Positive Control Checks

Tester strains were checked against the positive control substances twice: once when the 408 L concentrate was tested (Table 15a) and once when the Super Q and 20 L concentrates were tested (Table 14). In both tests all the strains reacted positively to a mutagen and the S9 preparation was shown to be active. The 2NF solution that was tested with the 408 L concentrate did not induce a positive response with strains TA 1537 and TA 98. Even though these two strains were shown to be sensitive to the mutagen 2AA suggesting that it was a faulty 2NF solution which lead to the non-positive response, the 408 L concentrate was retested on TA 1537 and TA 98 minus S9, as was a new 2NF solution. Upon retesting the 2NF produced a strong mutagenic response on TA 1537 (Table 15b).

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TABLE 14

Mutagenicity Test Results for the Fockler 20 L and Super Q Blank Water Concentrates. Each Value Represent the Average Revertant Colony Number from 3 Replica Plates.

Spontaneous Reversion Number (0) and Positive Control Data are Included.

Sample	Tester Strains (+ or - S9)							
	35		37		98		100	
	-	+	-	+	-	+	-	+
0	15	10	3	6	26	27	150	175
Positive Standards								
2NF (2 µg)					312			
(20 µg)			15					
NaN_3 (1 µg)	464						876	
2AA (1 µg)	12	178	3	34	19	719	181	815
Super Q Blank								
0.05 mL	25	18	2	3	21	30	177	178
0.20	24	22	1	4	20	21	197	158
20 L Concentrate								
0.05 mL	23	16	2	4	25	28	188	176
0.10	23	11	3	11	20	27	166	176
0.20	18	25	2	6	16	20	208	162

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TABLE 15a

Mutagenicity Test Results for the Fockler 408 L Water Concentrate.
Each Value Represent the Average Revertant Colony Number from 3 Replica Plates.

Spontaneous Reversion Number (0) and Positive Control Data are Included.

Sample	Tester Strains (+ or - S9)							
	35		37		98		100	
	-	+	-	+	-	+	-	+
0	13	14	2	5	13	24	154	143
Positive Standards								
2NF (2 µg)			5		12			
NaN_3 (1 µg)	153						328	
2AA (1 µg)	10	159	4	54	17	1248	135	1769
20 L Concentrate								
0.02 mL	28[1]	14	4	4	16	42	145	166
0.05	12	14	4	5	18	35	148	155
0.10	23	19	4	6	21	38	128	127
0.20	17	15	3	4	16	34	127	142

[1] Average of two replicate plates

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TABLE 15b

Repeat of Fockler 408 L Concentrate Using Strains TA 1537 and TA 98 in the Absence of S9.

Sample	Tester Strain (-S9)	
	TA 1537	TA 98
0	4	20
Positive Control		
2NF - 20 µg	77	
2 µg		860
408 L Concentrate		
0.02 mL	2	24
0.05 mL	4	20
0.10 mL	4	22
0.20 mL	4	18

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6. 20 L Concentrate

The results for the 20 L concentrate were very similar to those of the Super Q concentrate (Table 14). The data were indicative of a non-mutagenic response for TA 1537, TA 98 and TA 100, with a slight increase in the number of induced revertant colonies over that of the spontaneous for TA 1535. Since the TA 1535 results parallel that obtained with the pure water concentrate, this slight increase should not be considered significant.

7. 408 L Concentrate

Results of mutagenicity tests on the 408 L concentrate were indicative of a non-mutagenic response (Table 15a). There was one value (the lowest dose tested with TA 1535 minus S9) which was slightly high, however, no induced revertant colony number was two and one-half times higher than that observed for the spontaneous control (Table 15a). Since, as mentioned above, 2NF failed to produce positive responses with TA 1537 and TA 98 in the absence of S9, the 408 L concentrate was retested with these two strains (Table 15b). These results show 2NF mutagenicity on strains TA 1537 and TA 98 while at the same time confirming the non-mutagenic response of the 408 L concentrate on both strains (Table 15b).

OW 16-70

1. Toxicity

A toxicity check of the 20 L concentrate indicated no toxic effect to strain TA 98 cells (Table 16). This result was confirmed with visual monitoring of the bacterial lawn on all mutagenicity test plates.

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TABLE 16

Toxic Effect of the OW 16-70 20 L Water Concentrate on Salmonella TA 98 Cells

Volume of Concentrate Tested	Mean of Standard Error of Number of Surviving Colonies
	Dilution 1
0.00 mL	160 ± 5.7
0.05 mL	162 ± 5.0
0.10 mL	159 ± 6.9

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2. Spontaneous Mutation Rate

Although there was a noticeable inter-test variation with strain TA 100, all spontaneous reversion rates were within the normal range for the strain in question (Tables 17 and 18).

3. Super Q Concentrate

Test results for the pure water concentrate were clearly non-mutagenic (Table 17).

4. Reagent Concentrate

The results of the reagent concentrate were very similar to those of the Super Q concentrate (Table 17).

5. Positive Control Substances

In both positive control checks (Tables 17 and 18), the strains and S9 reacted as would be theoretically expected; all strains exhibited positive responses when exposed to the appropriate mutagen.

6. 20 L Concentrate

All four doses of the 20 L concentrate tested were non-mutagenic. The induced revertant colony counted did not signficantly exceed that of the spontaneous rate for the strain in question (Table 18).

7. 400 L Sample

Due to the difficulties and time involved in extracting large quantities of water from observation wells such as OW 16-70, which are underdeveloped deep wells lacking a system for pumping water out, no larger volume sample was taken.

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TABLE 17

Mutagenicity Test Results on the Super Q and Reagent Blanks Prepared Concomitantly with Samples from the OW 16-70 Well.
Each Value Represent the Average Revertant Colony Number from 3 Replica Plates.

Spontaneous Reversion Number (0) and Positive Control Data are Included.

Sample	Tester Strains (+ or - S9)							
	35		37		98		100	
	-	+	-	+	-	+	-	+
0	23	13	2	5	17	29	202	207
Positive Standards								
2NF (2 µg)					768			
(20 µg			38					
NaN_3 (1 µg)	580						1164	
2AA (1 µg)		185		7		1704		810
Reagent Blank								
0.05 mL	13	11	1	4	19	25	201	196
0.20	9	11	1	4	17	23	167	164
Super Q Blank								
0.05 mL	17	11	2	6	15	24	203	201
0.20	18	12	1	6	19	29	203	173

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TABLE 18

Mutagenicity Test Results for the 20 L Water Concentrate from the OW 16-70 Well. Each Value Represent the Average Revertant Colony Number from 3 Replica Plates.

Spontaneous Reversion Number (0) and Positive Control Data are Included.

Sample	Tester Strains (+ or - S9)							
	35		37		98		100	
	-	+	-	+	-	+	-	+
0	16	8	5	1	15	27	133	172
Positive Standards								
2NF (2 µg)					572			
(20 µg)			85					
NaN_3 (1 µg)	481						684	
2AA (1 µg)	22	72	3	34	17	453	160	892
20 L Concentrate								
0.02 mL	13	13	3	2	20	28	147	153
0.05	8	13	4	3	17	25	164	150
0.10	15	13	5	5	16	27	168	136
0.20	17	10	2	4	21	26	151	130

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DISCUSSION

The mutagenic analyses of the 20 and 400 litre well water concentrates from each of the four private wells and the 20 L concentrate from the on-site observation well were negative. This indicates that these well water samples, concentrated and extracted by current state of the art methodologies, were not able to produce a mutagenic response when tested on the Ames Salmonella/mammalian microsomal mutagenicity assay. The findings for the Ballantrae Plaza, Hutchinson and Fockler wells, which were previously tested, are consistent with the general conclusions of the previous report (November, 1982).

In the present study a large volume sample was taken from all but the OW 16-70 well. It should be noted that in selecting a volume of sample to be concentrated, the upper limit is often restricted because of practical consideration. The 400 L sample reflects the maximum practical volume which can be concentrated within one day with the system developed for this study. In the case of OW 16-70, an observation well lacking a water pumping system, 20 L was the maximum practical volume which could be sampled within a comparable time period.

Although there is no defined maximum volume which, when tested and found negative, would assure the absence of a mutagenic hazards, there can be little doubt as to whether the quantity of sample concentrated in this study was sufficient. The quantity of the large volume sample (approximately 400 L) taken from each well and the dose range tested approaches or exceeds that generally used in other mutagenicity studies on water. In addition, the toxicity to Salmonella observed with the Consolidated Gravel 404 L concentrate is a good indication that this sample, when tested, was at or near the maximum testable dose (MTD).

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Since toxicity is often brought on by the accumulation of mutagenic lesions in an organism (the reverse is not true), testing at the MTD should be sufficient to detect mutagenic compounds, if they are present. In those cases where the toxic effect is independent of a mutagenic event, it would be possible, albeit unlikely, for a test substance to be determined mutagenic if tested beyond the MTD. For the most part, however, the mutagenic result would be undetectable as the test organism would die due to the toxic effect before the mutagenic event could be expressed. In this case, toxicity to the test organism, not mutagenicity, would be the prime concern.

Of the five wells tested, OW 16-70, because of its location on the landfill, was thought the most likely to produce a positive response in the Salmonella assay. However, like the 20 L concentrates from the four private wells, the OW 16-70 20 L concentrate also elicited a non-mutagenic response. These results suggest that if there were any organics present in OW 16-70, they were not mutagenic or else not present in a high enough concentration to induce genetic reversion in the Salmonella assay. A 1981 sample from OW 16-70, which was chemically analyzed, indicated the presence of several organic compounds one or more of which could possibly be mutagens. These analyses further indicated, however, that the concentrations of the organic compounds were generally less than 1 µg/L; probably below the detection limits of the Ames test even when concentrated from 20 L of water. Since inherent quality control measures indicated that the Salmonella test system was sensitive to mutagens, the negative response with the 20 L OW 16-70 sample probably reflects the absence of mutagens or, their presence in a concentration too low to induce an effect in the test system. In either case the concentrate is considered non-mutagenic.

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In most cases the quality assurance tests on each well checked out as expected. The tester strains generally responded normally to positive and negative control substances, and the S9 activation system, in all but a few instances, proved to be functioning. Variations in the responses on the Ames test observed with the Super Q and reagent blank concentrates appeared to be within a normal range and reflected variations in the spontaneous reversion rate or S9 activation mixture. The overall level of quality assurance was very high and this further confirmed the validity of the non-mutagenic results obtained with the test concentrate. In those few identified instances where deviations from the quality control checks were observed, repeat testing of both the quality control and the test substances were conducted. These repeat tests confirmed the proper condition of the test as well as the original finding for the test substances which in every case were non-mutagenic.

The test results on all of the 20 L concentrates reported here confirm previous MOE testing of 20 L concentrates from three of the wells reported for November 1982 in the Whitchurch-Stouffville mutagenicity study. In both studies the concentrates were non-mutagenic. In particular, this present study confirms with both the present 20 L and 400 L samples the previous non-mutagenic data obtained with 20 L concentrates. The main difference in the two studies is that the present samples were taken in October rather than January-February as was the case for the previous study.

The findings in this report again do not corroborate the original mutagenicity findings of Dr. J. Cummins reported for an October 1981 Hutchinson well water sample.

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The lack of a positive mutagenic result in any of the three wells from the previous study makes it difficult to evaluate any possible seasonal effect. To date three of these wells have been sampled and tested in two seasons. The results of tests on samplings in both seasons have been non-mutagenic. As discussed in the November 1982 report, a true evaluation of seasonal variation would require a well water source which produces a positive mutagenic effect in at least one season and which has been sampled seasonally for at least two years. The findings in this study, however, do indicate that the differences in the results reported by Dr. J. Cummins for the October 1981 Hutchinson well water samples and those reported both here and in the November 1982 study are probably not associated with seasonal variations. If the differences were a product of seasonal variation, the 20 L and 447 L samples reported here would have been expected to produce Ames assay positive results.

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CONCLUSIONS

Well water from five wells in the Whitchurch-Stouffville area was sampled, concentrated and extracted by current state of the art technology. A 20 L concentrate was prepared from all wells and a 400 L (approximate volume) concentrate was prepared from all but the observation well, OW 16-70.

Ames assay test results on all concentrates proved negative for mutagenicity suggesting that these waters, at the concentrations tested, are not capable of inducing mutations in the Salmonella assay.

The results of this study confirm those reported in the November 1982 Whitchurch-Stouffville mutagenicity study. The 1981 report of Dr. J. Cummins indicating that the Hutchinson well water contained substances capable of producing a positive Ames mutagenicity response again could not be substantiated.

Because the Hutchinson well water sample taken for this study was taken in the same month as that for the 1981 study, it is unlikely that the discrepancies in the findings for these two studies can be attributed to seasonal variation.

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APPENDIX

1. Standard Operating Procedures for Plate Incorporation Assay.

2. Standard Operating Procedures for Maintenance of Tester Strains.

STANDARD OPERATING PROCEDURES FOR PLATE INCORPORATION ASSAY

Date: June 1, 1982 Date Superseded: _____

1. If needed, the protocol is described in the following article:

   Ames, B.N., J. McCann and E. Yamasaki, 1975, 'Methods for Dectecting Carcinogens and Mutagens with the Salmonella/Mammalian Microsome Mutagenicity Test', Mutation Research 31: 347-364. A copy of this work as well as other on this protocol are kept in the Biohazard Unit file.

2. Preparation for experiment;

   a. Prepare outline of experiment indicating the number or amount of the following items required:

      1) Minimal agar plates,
      2) Nutrient agar plates,
      3) 13 x 100 mm sterile tubes and caps,
      4) Top agar,
      5) Histidine and biotin (H&B),
      6) S9,
      7) The test chemical at each dose.

      Where practical, be sure to allow an additional 1-2 ml of S9 in order to compensate for mistakes and losses due to pipetting. Similarly, prepare extra test chemical at each dose to allow for errors, and difficulties in retrieving the entire quantity prepared from the containers.

   b. Prepare a diagram of how the experiment is to be set up in the dry block. Designate which tubes, columns or rows are to contain specific strains, individual doses of test chemical, and S9.

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c. Check on availability and quantity of the actively growing cells. Since a minimum of six hours is required for the preparation of cells, care should be taken the day before the experiment to insure an adequate supply of the appropriate tester strain.

d. Place containers with the required amount of top agar into a glass or metal flask containing water, then bring the water to boiling. Allow agar to completely melt, i.e., the resulting solution should be totally clear.

e. After the top agar has melted it should be transferred to a 50-55°C water bath. After the agar has cooled to below approximately 60°C, add enough H&B to bring the final H&B volume to 10%. (It is necessary to have top agar at 50-55°C, at this point, rather than 42°C, because there will be some cooling when the H&B is added and the top agar will cool further during the time required to 'load' the 13 x 100 mm tubes).

f) Place, according to the outline and the diagram, the appropriate number of sterile, capped 13 x 100 mm tubes into dry temperature blocks. These blocks are to have been set at 42°C. Once the block has reached 42°C, it must remain at that temperature for the duration of the experiment.

g. After the temperature blocks have been maintained at 42°C for at least ½ hour, 2 ml of the minimal agar (now containing 10% H&B) is added to all appropriate tubes. Another approximately 15 minutes is now required for top agar to stabilize at 42°C.

h. Where required, 2 ml of nutrient agar is added to the appropriate tubes.

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i. All the plates required for the experiment are to be coded and assembled near the dry blocks. (in cases where many plates are required, the plates can be coded the previous day if the experimental outline has been prepared).

j. The code for each plate is to include the following:

1) Experiment number which is the date, e.g., 110582,
2) Test compound -- if more than one is used in the experiment,
3) Dose of test compound added to plate,
4) Presence or absence of S9,
5) Tester strain used, if more than one per experiment.

k. Disposable plastic bags should be taped onto the edge of the work bench into which mutagenic waste material, i.e., pipets or tips used to handle test compounds, spent gloves, 13 x 100 mm tubes and caps, etc. are placed.

l. For pipets not used for test chemical transfer, a container partially filled with a bactericide should be used as a receptacle.

m. The S9 solution should be prepared at the last possible moment. The buffer may be prepared earlier and then kept on ice, but the liver homogenate should only be thawed and added just prior to use.

3. Experimental procedures;

a. Into the tubes of top agar and H&B are to be put:

1) Test chemical,

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2) Cells,

3) S9, if required.

b. The sequence of addition should be as presented in a. above.

c. Each appropriate dose and amount of test chemical is to be added to each tube. The loading of the test chemical should take place in either a fume hood or glove box. A new pipet or tip is to be used with each dose. Care should be taken to load the chemical into the tube either without touching the inside of the tube or as close as possible to the level of the agar in order to avoid cross-contamination during subsequent operations.

d. Depending upon the heat stability of the test chemical, it may be added to either one or all the tubes within a block. Most test chemicals can easily withstand 42°C for the time necessary to complete the test, thus the chemical can be loaded into all 20 tubes within a given block.

e. 0.1 ml of the appropriate tester strain is to be added to each tube. In order to avoid the pipet carrying test material from a high dose tube into a low dose tube, cells should be added to the tubes with the lowest dose of test chemical first. These tubes are then mixed vigorously using a vortex. The subsequent addition of cells should then proceed to the tubes with the next lowest dose of the chemcial. The procedure is repeated until all tubes within a given test group have been loaded with cells and the tubes vortexed.

f. If no activation mixture is to be added, the tubes are poured immediately. With two people working an experiment, each tube is to be poured as soon

as the final ingredient (in this case the cells) is added. With only one person, tubes should be poured after a given set of tubes has been loaded. The set should not exceed 15 tubes.

g. When S9 is required, 0.2 ml of the final solution is added to each tube. Again in order to prevent carry-over of test material or cells, the first tubes to which S9 is added should be those which contain the tester strain with the lowest spontaneous revertant number. Within that group, tubes with the lowest dose of test substance would be first. The S9 is then added to the next highest dose with the given tester strain and so on until all tubes with that strain have been loaded. The pipet is then discarded.

h. With a new pipet S9 is then added in similar fashion to those tubes containing the strain with the next lowest spontaneous revertant number.

i. Tubes are poured as discussed in f. above.

k. The poured plates are then incubated at 37°C for 40-48 h and then the number of his^- revertants counted.

STANDARD OPERATING PROCEDURES FOR MAINTENANCE OF TESTER STRAINS

Date: June 1, 1982 Date Supersided: _____

A. Tester Strain

1. Tester strains in the laboratory:

   a. TA 1535 - A his^- G46 mutated strain used in the detection of mutagens inducing base-pair substitution.

   b. TA 1537 - A his^- C3076 mutated strain . Used in the detection of mutagens capable of causing frameshift mutagen.

   c. TA 1538 - A his D3052 mutated strain. Used to detect mutagen causing frameshift mutagen.

   d. TA 98 - TA 1538 into which a pKM101 episome has been added. A strain more sensitive in the detection of frameshift mutagen than TA 1538.

   e. TA 100 - TA 1535 into which the pKM101 episome has been introduced. Provides a more sensitive system for detecting base-pair substitution than can be obtained with TA 1535.

2. In general, either 3, 4 or 5 strains are used in the testing of an unknown. Since TA 98 and TA 100 are more sensitive than the strains from which they were derived (TA 1538 and TA 1535, respectively), tester strains TA 1535 and TA 1538 may be eliminated from the test protocol. However, there is some

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there is some evidence that TA 1535, due to its low spontaneous reversion rate, may be able to distinguish certain chemicals as mutagen which may be missed by TA 100. Thus TA 1535 is often used along with TA 100.

3. For testing an unknown, four of the strains will be used routinely. These are TA 1535, TA 1537, TA 98 and TA 100.

B. Storaging Stock Supply of Strains

1. To insure a perpetual supply of cultures with appropriate markers, cells are frozen in DMSO and stored in -80^{o}C freezer in Room E-422.

2. To freeze cells, take 0.8 ml of a logarithmically growing culture and place in a 2 cc sterile glass screw top vial which has been previously labeled and color coded as to strain classification, data and set number. To the cells add 0.07 ml of spectrophotometric quality DMSO and freeze.

3. For each strain at least two sets should be prepared. One or more sets will serve for routine use while one set will be set aside as a master colony, only to be used in case of emergency and than only to generate additional sets.

C. Preparation of Master Plates

1. New master plates should be prepared approximately every four weeks, although these plates can be used for up to 2-3 months.

2. Master plates are used for routine work in order to avoid the problems which arise when frozen culture are opened frequently, i.e. surface

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thawing and refreezing can damage bacteria, and it is not always easy to obtain adequate inoculum from frozen vial.

3. Using a sterile probe, i.e. wooden stick or wire loop, transfer inoculum from frozen culture to a 8 x 150 mm tube containing 5 ml of nutrient broth. Repeat for all 5 strains.

4. Grow cultures overnight or, if inoculums taken in morning, until sufficient growth has occurred to make broth appear cloudy (4-6 h). With tester strains TA 98 and TA 100, 0.05 ml of a 8 mg/ml ampicillin solution is added to the broth.

5. With sterile wire loop, streak culture onto nutrient agar plate.

6. Incubate overnight at $37^{o}C$.

7. Pick isolated colony from plate and transfer into 18 x 150 mm tube containing 5 ml of nutrient broth. Three colonies should be isolated from each plate.

8. Incubate 6^{+} h on $37^{o}C$ shaker.

9. Test each of the cultures for the following markers:

   a. R-factor

      1) the pKM101 episome in strains TA 98 and TA 100 may be loss with successive transfers of those cultures. Since the episome, beside increasing the sensitivity of the strain, provides an

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ampicillin resistant marker, it is possible to easily monitor for the presence of the episome by challenging the culture with ampicillin.

2) A 0.1 ml of an 8 mg/ml ampicillin solution is streaked down the center of two nutrient agar plates. The streaks are allowed to dry and then the cultures are each cross-streaked against the ampicillin. Or, cultures are cross-streaked first and a sterile ½" strip of filter paper is placed vertically across the streaker. Then 0.1 ml of an 8 mg/ml ampicillin solution is used to wet the filter paper.

3) One plate is for the three TA 98 colonies and the other for the TA 100 colonies.

4) Be sure to label both the tube and its corresponding streak.

5) Each plate should also contain one streak of either TA 1535, TA 1537 or TA 1538 as a control.

6) Incubate the plates overnight at 37°C.

7) Check for toxicity. The streak from cultures which have lost their R-factor will appear clear near the area of the ampicillin.

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b. rfa mutation -

1) In the development of each strain, a portion of the lipopolysaccharide membrane has been removed to decrease the cell's selective permeability barrier. Thus test chemicals will more easily be absorbed by the cell.

2) To check for continuous loss of the lypopolysaccharide membrane, the cells are challenged with a 1 mg/ml crystal violet solution.

3) The cell cultures are streaked horizontally across nutrient agar plate. Again care should be taken to note which streak came from which culture tube.

4) A ⅛ - ½" strip of filter paper is placed in the centre of the plate vertically crossing all the culture streaks.

5) Approximately 0.1 ml of the crystal violet solution is down the filter paper strip.

6) Plates are incubated overnight at 37°C.

7) A clear (toxicity) zone near the crystal violet strip indicate the presence of the rfa mutation, which permits large molecules like crystal violet to enter the cell and inhibit growth.

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c. Histidine requirement

1) Spread 0.1 ml of a 0.5 mM biotin solution onto 10 minimal agar plates.

2) Spread 0.1 ml sterile 0.1 M L-histidine onto 5 of these minimal agar plates.

3) Streak cultures (1 tester strain per plate) onto the 5 histidine supplemented plates and the 5 non-histidine supplemented plates.

4) Incubate 40-48 h at 37°C.

5) Check for a growth differential between histidine and non-histidine supplemented plates.

d. UVRB deletion

1) Streak cultures (1 tester strain per plate) onto 5 nutrient agar plates.

2) Irradiate half of the streak for 8 sec with a G.E. 15 watt germicidal lamp at a distance of 33 cm.

3) Incubate overnight at 37°C.

4) Irradiated portion of streak should show no growth.

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e. Spontaneous reversion rate

1) Set up 13 x 100 mm tubes with 2 ml top agar, minimal agar plates, and S9 as described in Procedures for Plate Incorporation Assay.

2) Three to five replica plates should be prepared for each culture, both with and without S9, i.e. 15 cultures x 3 replica x with or without S9 = 90 plates.

3) Add 0.1 ml of the appropriate cell culture to the tube, pour or, if required, add 0.2 ml of S9 and then pour as described in Procedure for Plate Incorporation Assay.

4) Incubate 40-48 h at 37°C.

5) Count the number of revertant colonies. Compare to standard reversion rates for each strain, which are as follows:

Strain	Range of Reversion Rate	Ideal Number
TA 1535	10 - 35	20
TA 1537	3 - 15	7
TA 1538	15 - 35	25
TA 98	20 - 60	40
TA 100	120 - 250	160